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TITLE: Gene-Probe Electrodes to Detect Enterically-Transmitted
RNA Virus Pathogens

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13. ABSTRACT (Maximum 200) Viral infections are the most common cause of human disease and are responsible for at least 60% of the illnesses that cause physician visits. These organisms are difficult to detect as complex tests either take days to generate accurate results or lack sensitivity, specificity, and quantitative capability. AndCare reports the demonstration of a new electrochemical approach to nucleic acid based detection that can rapidly detect, identify and quantify viruses in crude (unpurified) samples. This same system can be used as a simple, quantitative detector for systems such as reverse transcriptase polymerase chain reaction (RT-PCR) that amplify viral nucleic acid sequences. Proof of principle was obtained by modifying AndCare's disposable colloidal gold electrodes to generate a current when a hybrid is formed between a gene probe and the specific RNA sequence of a target virus. We have shown direct detection of PT-PCR product of polio virus at levels as low as 5 plaque forming units (pfu) of virus taken from a tissue culture sample containing 5×10^4 pfu/mL. This is a breakthrough in viral research, since our methodology can speed viral detection, identification and quantification, and thereby has the potential to revolutionize health care related to viral disease.				
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FOREWORD

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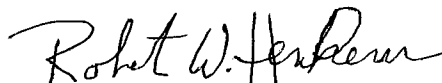
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1.0 INTRODUCTION

Viral infections are the most common cause of human disease and are responsible for at least 60% of the illnesses that cause visits to a physician (Ray, 1979). Unfortunately, these organisms are also difficult to detect, requiring complex laboratory tests that either take days to generate accurate results (e.g. culture), are inadequate in sensitivity and specificity (e.g. monoclonal antibodies), or lack quantitative capability. We have now demonstrated that our new electrochemical approach to nucleic acid sequence detection can be used to rapidly detect, identify and quantify viruses in crude (unpurified) samples and that this same system can be used as a simple, quantitative detector for systems such as reverse transcriptase polymerase chain reaction (RT-PCR) that amplify viral nucleic acid sequences.

The time, expense, and expertise required for tissue culture isolation for viruses has limited viral diagnostic services to a handful of medical centers and reference laboratories. Antibody assay for viral disease are simpler to perform but antibodies to the virus are often not detectable until well after onset of disease. Recent advances in molecular biology have enabled detection of specific DNA or RNA sequences and have led to development of highly selective assays for detection for a variety of nonculturable or difficult to culture viruses such as members of the enterovirus family (Robart, 1990) including hepatitis A, B, and C (Jansen, et al., 1990; Kaneko, et al, 1989; Ichimura, et al., 1992). The new molecular biology advances are dramatic improvements, but the procedures involved are complex and time consuming, requiring experienced operators and use of systems like the polymerase chain reaction (PCR) that amplify DNA or RNA to levels that can be detected. Until now it has not been possible to easily detect the product of the amplification process, nor to easily and directly detect, identify and quantify levels of specific DNA and RNA sequences without amplification or use of radioactive labels. AndCare's electrochemical technology enables simple, direct and quantitative detection of DNA and RNA sequences characteristic of viral pathogens. In our system, the current generated when complementary gene probes and targets hybridize provides proof that specific DNA or RNA from a organism is present in a test sample. The amount of current generated is a measure of the quantity of the DNA or RNA that is complementary to the probe sequence.

The genus *Enterovirus* includes long established human pathogens such as polio virus, hepatitis A virus, human rotavirus, coxsackievirus, echovirus and newer enteroviruses such as Hepatitis E viruses. The genus *Enterovirus* contain a large number of distinct serotypes that cause diseases ranging from mild to life threatening. Major pandemics caused by various strains of rotaviruses, a genus within the family Reoviridae, result in staggering levels of death from diarrheal illness in infants in developing countries, with total infections estimated at 450 million yearly with a 1 to 4 percent fatality rate (Fields Virology). Hepatitis E viruses, recognized only since 1988 as the cause of epidemic human liver disease, are common throughout topical zones of Asia, Africa, and South America. Illness associated with this virus was previously classified as non-A, non-B hepatitis. These and other viral pathogens such as dengue virus are of great concern to human health, since in many cases the diseases they cause can be transmitted from one to many individuals. Our work to date has used representatives of the genus *Enterovirus* to provide proof of principle for a new electrochemical procedure for detection of these and other pathogenic viruses.

2.0 OVERALL PROGRESS IN PHASE I RESEARCH

We have obtained convincing evidence that our innovative electrochemical approach to detection of nucleic acid sequences can be used to detect and quantify the presence of specific RNA viruses in crude samples such as blood at clinically relevant levels. Proof of principle was obtained by modifying our disposable colloidal gold electrodes so that they generate a current when a hybrid is formed between a gene probe and the target sequence specific to the RNA of a particular virus. We have also shown direct detection of PT-PCR product of polio virus at levels as low as 5 forming units (pfu).

High selectivity and rapidity of testing was obtained by use of a gene-probe hybridization technique with the AndCare electrochemical detection system. When used with our disposable sensors, this technique allows for detection, identification and quantification of nucleic acid sequences characteristic of specific viral pathogens in crude samples. Following proof of principle of our electrochemical approach to viral detection described below, we have carried out further research on the complete AndCare detection system (monitor, sensor, reagents and procedures). We have made good progress (described in the following sections) in our efforts to:

- Design, synthesize and evaluate DNA-probes with electroactive reporter groups for detection of our Phase I target viruses (hepatitis A virus and polio virus),
- Generate disposable and inexpensive electrochemical DNA-probe biosensors with colloidal gold electrodes that make use of the electroactive gene-probes,
- Tailor the electrochemical monitor design for rapid, facile and quantitative detection of the sandwich hybrids formed between the DNA-probes and the target nucleic acid sequences,
- Culture polio virus to provide material that facilitates tests of the system,
- Show electrochemical detection of hepatitis A virus nucleic acid sequence,
- Show electrochemical detection of virus-specific complementary DNA clone (cDNA), and
- Show electrochemical sensitive and selective detection of polio virus.

Our progress has been aided by Dr. Valentina Kazantseva, a new member of the AndCare team. She is experienced in the culture, concentration and detection of polio virus and has used this capability to aid viral studies in conjunction with this project. We asked her to first culture polio virus, since we wished to verify reports of a sensitive type of gene probe for polio virus, potentially adaptable to our AndCare technology, that was presented in the literature (Bosch, et al., 1995). This proved to be successful. Probes like those described by Bosch, *et al.*, allowed us to detect virus-specific complementary DNA (cDNA) without optimization of the system and to detect polio virus grown in tissue cultures at clinically relevant levels down to 5×10^3 pfu/mL. We consider this a breakthrough in viral research, since our methodology can speed viral detection, identification and quantification, and thereby has the potential to revolutionize health care related to viral disease.

3.0 MATERIALS AND METHODS

Virus strains

The virus strains used in this research were polio virus (PV) type 1 strain OPV (Sabin), PV type 2 strain OPV (Sabin) and PV type 3 strain OPV (Sabin). These were obtained from Division of Product quality control CBER/EDA of the Food and Drug Administration (Rockville, MD, USA). Another strain, PV type 1, strain LSC2ab (Sabin) was obtained from the World Health Organization (WHO) reference laboratory of the Institute of Poliomyelitis and Viral Encephalitis (Moscow, Russia).

Virus culture lines

Hep-2 (human larynx epidermis carcinoma, obtained from the Tissue Culture Department, University of North Carolina, Chapel Hill, NC) and Vero (African green monkey kidney, obtained from Division of Product quality control CBER/EDA of the Food and Drug Administration (Rockville, MD, USA) cell lines were used for growth and maintenance of polio virus. They were maintained according to the suppliers' recommendations. Briefly, cells were grown in cell culture medium with 10% calf serum added. 0.05% trypsin-EDTA was used to detach cells from the culture vessel. In general, a subcultivation ratio of 1:4 was used for cell passage in fresh media.

Virus culture methods

PV was cultured according to standard protocols (Manual for the Virological Investigation of Poliomyelitis, World Health Organization Expanded Programme on Immunization and Division of Communicable Diseases). Briefly, virus was propagated at 37 +/- 0.5C on healthy confluent monolayers. Cells were washed one time in Dulbecca's Modified Eagle Media (DMEM) without serum before inoculation. Approximately 10^{6-7} TCD₅₀ were used for inoculation. PV were isolated after 15 - 17 hours of subsequent culture at the standard temp.

Titration of PV stocks was accomplished by two methods. In the first case, cytopathic effect (CPE) was determined by inoculation of cell cultures with various dilutions of live virus. Titer is expressed as TCD₅₀, and is defined as the highest dilution giving CPE in 50% of inoculated cell cultures. The second method involved identifying and counting plaques in inoculated cell cultures under a layer of media solidified with agar. The cell monolayer is washed with DMEM without serum. Virus dilutions were dropped gently onto the monolayers and allowed to adsorb for 20 - 30 minutes. Agar media was prepared { 140.0 mL DMEM (70%), 10.0 mL calf serum (5%), 1.0 mL neutral red (1:30,000 dilution) ($1.7 \times 10^{-5}\%$), 50.0 mL 2.5% Difco agar (0.62%) } 5.0 mL of 37 C agar media was poured gently on top of the monolayers. Plates were protected from light, incubated at 36 C in air + 5% CO₂. Plaques were counted from 3 to 7 days. Virus titer is presented as plaque forming units (PFU) per mL.

Polio culture has made use of Hep-2 (human larynx epidermoid carcinoma) and Vero (African green monkey kidney) cells that have now have been grown in culture and infected with polio virus (Sabin Type 1, LSc 2ab). These have yielded viral titer values ranging from 10^6 to 10^9

TCD₅₀/ml. Substantial amounts of polio virus have been isolated from these cultures. This virus is now routinely in our polio virus detection assays.

Polio virus processing

Several lysis procedures have been tested for the polio virus. We sought a procedure that denatures the polio virus, allows for probe hybridization and does not interfere with our electrode chemistry. Several lysis methods were tested (detergent, guanidinium, commercial RNA isolation kit, and freeze-thawing). Freeze-thawing for 3-5 cycles in dry ice/water bath appears to best meet the above criteria with minimal requirements in terms of time, manipulation or equipment. This combination of culture and lysis was used in our direct detection of the Sabin 1 polio virus in tissue culture media.

Culture of other viruses

We have initiated cultures of cell lines designed to grow both hepatitis A virus and rotavirus. These cell lines, FRhK-4 (fetal kidney, rhesus monkey, ATCC no. CRL-1688), Vero (kidney, African green monkey, ATCC no. CCL-81), and MA-104 (African green monkey kidney, BioWhittaker, Walkersville, MD) have been in culture at AndCare for several weeks.

Initial Viral Nucleic Acid Probes

A fluorescein-labeled detector probe and 5'-biotinylated capture probe with sequences complementary to that of hepatitis A were synthesized. A 62 nucleotide (nt) artificial DNA target was also synthesized corresponding to bases 333-395 of hepatitis A RNA.

Initially probes for detection of polio virus were based on a paper by Bosch, et al. These large probes (up to 1400 bases long) were multiply labeled with digoxigenin (DIG). Additionally, we designed and used two sets of two small probes to be complementary to the polio virus genome. Detector probes were complementary to nt 601 - 624 and 651 - 670 (DIG labeled) and 3431 - 3461 and 3363 - 3393 (Fl labeled). The corresponding capture probes were complementary to nt 677 - 706 and 3398 - 3427. The use of these short probes with the single stranded RNA isolated directly from polio virus did increase the sensitivity and specificity of the reaction, as described in the following sections.

Electrochemical Detection via Redox-Active Probes.

AndCare technology relies on the electrochemical properties of enzymes or other redox-active molecules for detection and quantification of nucleic acids. These can be intrinsic to the substance being detected or extrinsic labels. A requirement for electrochemical nucleic acid sequence detection is that there be a redox-active material captured at the working electrode. In use, DNA or RNA for analysis is exposed to labeled probe(s) that hybridize specifically to the target DNA or RNA. After hybridization, the modified colloidal gold electrode is brought in contact with the sample. The hybrid binds to the electrode, using for example a biotin labeled probe and a streptavidin modified colloidal gold electrode. Other binding pairs may be used. A second probe is used which contains a second label such as fluorescein. Enzyme conjugated to an antibody

directed against this second label is added, incubated, washed and followed by addition of enzyme substrate and a mediator. If hybridization has occurred, the enzyme present in the hybridized DNA will generate electrons when an appropriate voltage is applied. These electrons are carried to the electrode by the mediator and a current is generated only when the target is present to form a bridge between capture probe and detection probe (Figure 1).

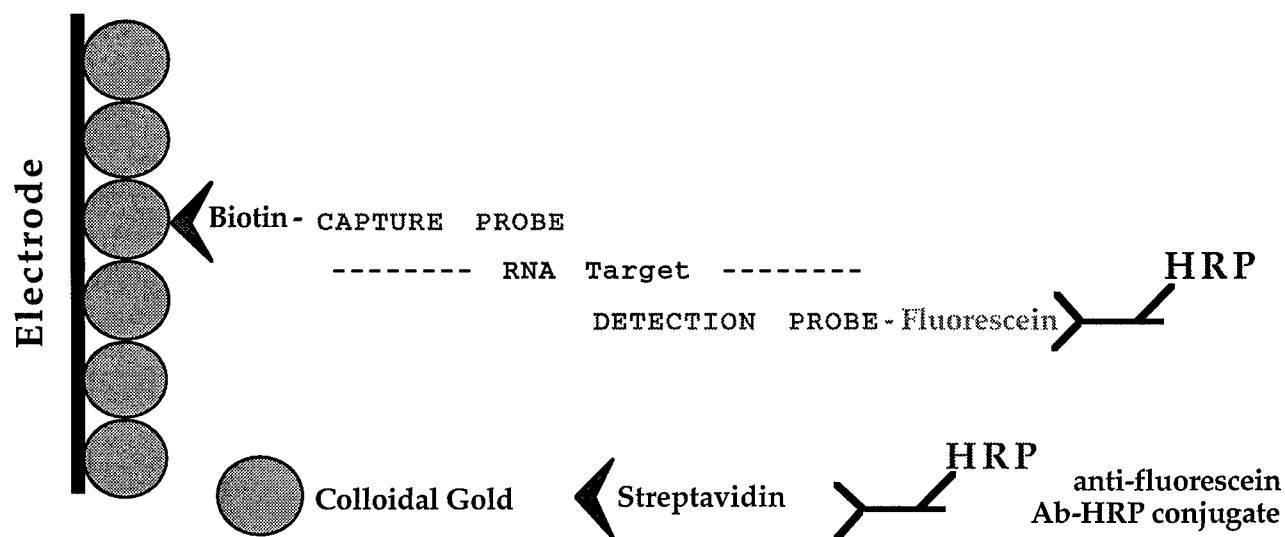


Figure 1. Redox-active nucleic acid hybrid captured at colloidal gold modified electrode.

Catalytic current is produced by mediated electron transfer between the enzyme and the electrode which is measured by the monitor as schematically illustrated for HRP in Figure 2. We will study as described in the following sections the effectiveness of the electrocatalytic reaction with different mediators, enzymes and substrates.

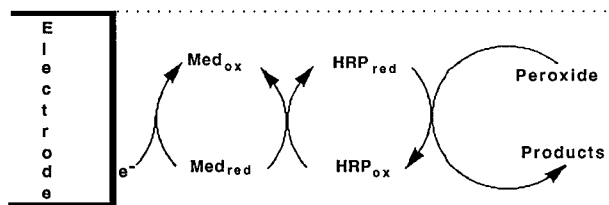


Figure 2. Production of catalytic current from HRP reaction in the presence of substrate and a mediator (Med).

Equations 1-3 illustrate reaction sequences we have utilized for the electrochemical detection of hybrids containing an electroactive group (Henkens, et al., 1997; Henkens, 1996; Zhao, et al., 1996; Crumbliss et al., 1993; O'Daly et al., 1992; Zhao et al., 1992; Henkens et al., 1992a; Henkens et al., 1992b). In this example an enzyme label HRP is used but, as noted, we will use non-enzymatic electroactive groups in the proposed work. Good results are obtained using guanine as the electroactive reporter, a base which is intrinsic to the nucleic acid target. An electron transfer mediator is used with both intrinsic electroactive groups and redox-active labels added to the detector probes. Figure 3(a) illustrates the cyclic voltammogram obtained for the electrode in

background electrolyte at pH 7.0 in the presence of ferrocenecarboxylic acid as a mediator. Figure 3(b) illustrates the generation of a catalytic reductive current for the same system with the addition of H_2O_2 . Measurement of the catalytic current is made at fixed potential. The catalytic current at excess mediator is directly proportional to HRP, which is in turn proportional to the level of captured probe-target.

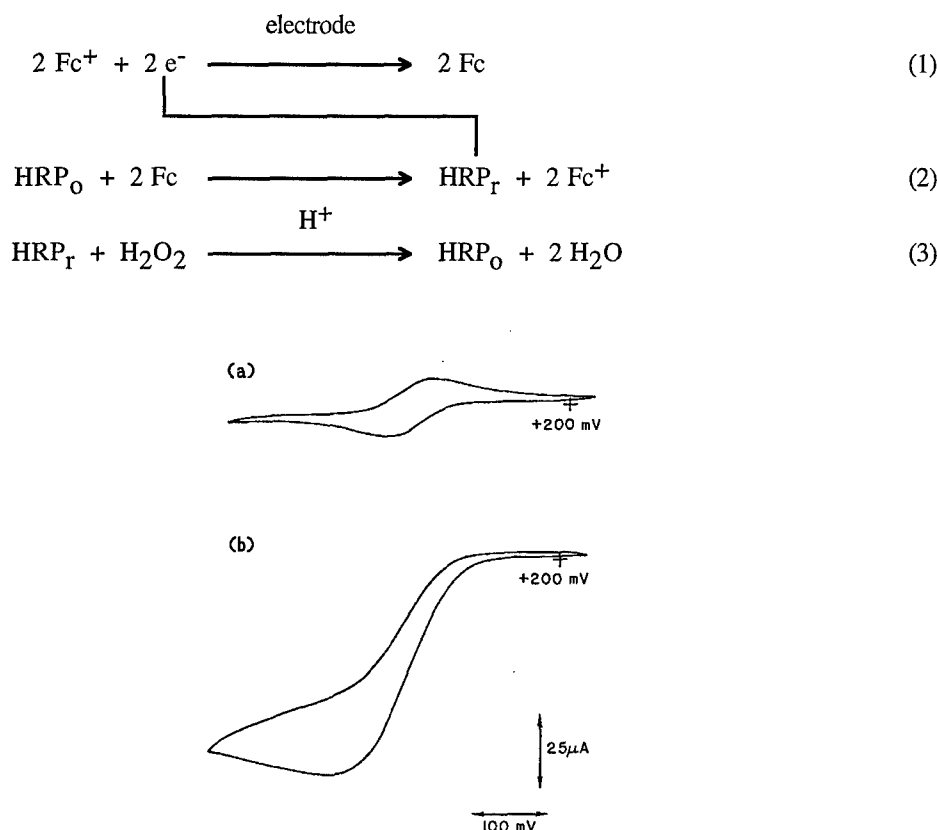


Figure 3. Cyclic voltammograms for HRP/colloidal gold on a carbon electrode as described in Eqs. 1-3., in the absence (a) and presence (b) of 4 mM H_2O_2 .

AndCare technology can be used with a range of redox-active compounds or enzyme labels on the probes. Alternatively it can work without an added redox-active compound, making use of the fact that it is possible to electrochemically detect guanine, a redox-active constituent of nucleic acids. In either case, the target nucleic is allowed to hybridize with one or more probes in solution. The hybridization occurs more rapidly in solution than onto an immobilized probe, lessening the required time per test. Current is only generated for target-probe hybrids captured at the electrode surface and for this to occur, specific hybridization between target and probe(s) and electrode capture must occur. This can give great specificity to the system. As shown in the results section, a single base pair mismatch can be detected.

Gene-probe biosensors

We generated disposable and inexpensive electrochemical Gene-probe biosensors for detection of viruses by screen printing. The electrodes are printed using a polyester screen using conducting

inks supplied by DuPont. Multiple overprintings of dielectric are used to build up the well to contain the sample. Deposition of colloidal gold onto a sheet of 40 screen printed sensors is accomplished with microdeposition equipment as shown in Figure 4. Individual sensors are then die cut from the sheet. The high sensitivity of our biosensors derives from a microelectrode array of gold particles fabricated by deposition of colloidal gold on the surface of the working electrode (central black circle on each sensor). The microelectrode array generates current when used with the redox-active gene-probes and capture techniques described above.

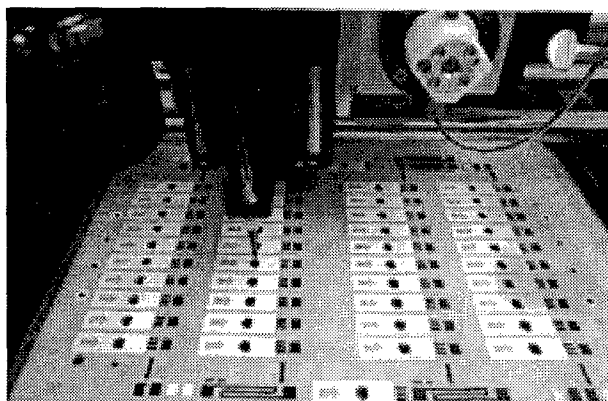


Figure 4. Picture of controlled deposition of nanometer sized colloidal gold particles onto screen printed carbon electrodes.

Gold trichloride ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$) (Fisher Chemical Co.) was used to prepare colloidal gold sols. These sols were modified with neutravidin rather than streptavidin to reduce non-specific cell binding, while retaining high binding affinity for biotin. The neutravidin-colloidal gold sol was concentrated, collected, resuspended in buffer and then deposited on the carbon working electrodes to make the cAu-modified working electrode of the sensors. These printed carbon electrodes, coated with colloidal gold/neutravidin, are now routinely used to detect polio virus in our AndCare system.

Electrochemical Measurement by Hand-Held Monitors

The AndCare electrochemical monitoring system makes use of a monitor that can perform square wave voltammetry, various pulse methods, or differential amperometric measurements as needed. It operates as a stand-alone unit with LCD display, powered by a battery or DC power supply, or as a computer interfaced system with increased flexibility (for changing parameters, uploading current curves and test results, and storing data for later analyze) using a built-in RS232 interface for the computer connection or printer connection.

4.0 RESULTS

The AndCare assays are based on a principle of electrochemical detection of specific nucleic acid sequences using a method invented by us. This electrochemical methodology makes it possible to probe a complex sample and quantitate the presence of a specific nucleic acid sequence within it. It provides a new way to approach the design and development of sensors that can detect certain nucleic acid sequences at relevant. The potential impact of this simple and inexpensive technology on biomedical research and diagnostic development is far reaching.

Monitor/Sensors used for Electrochemical Detection of Viruses

The results which follow make use of the basic principles of this novel electrochemical nucleic acid detection technology described in the Phase I proposal and briefly restated here. We designed, tested, and are improving a new, simple to use electrochemical monitor for conducting viral tests using AndCare's disposable sensors. The monitor and sensors were tested with hepatitis A synthetic target, polio cDNA and cultured polio virus.

This system for viral detection builds on the platform developed for detection of RNA sequences. The monitor and associated printer used in our viral assays are shown in Figure 5. The monitor has a port for sensor insertion at lower right of the casing.



Figure 5. AndCare monitor and associated printer used in viral assays.

The operation procedure is shown in Figure 6.

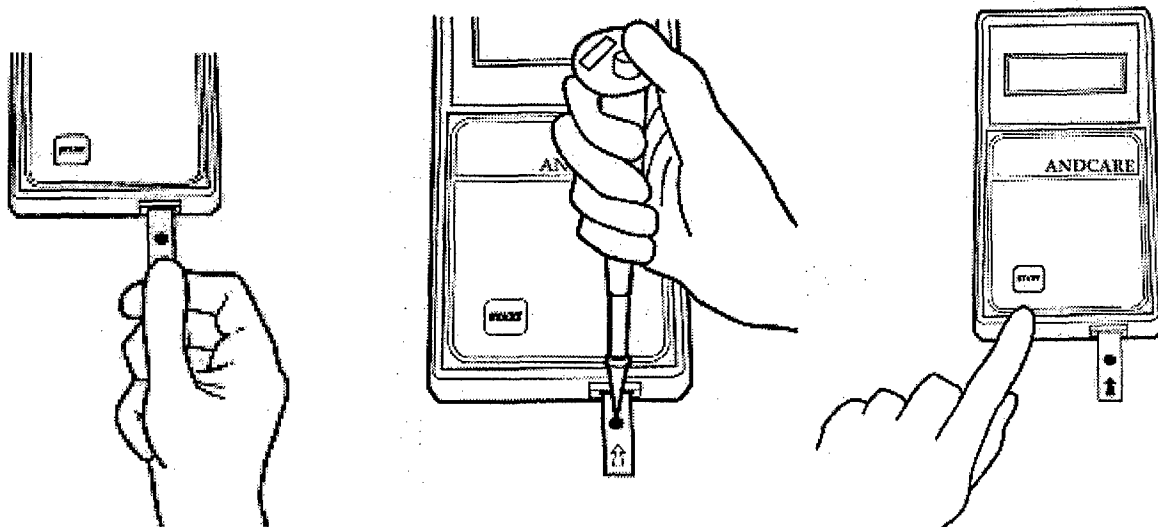


Figure 6. Illustrated operation of the ANDCARE Monitor.

The electrochemical detection of specific viral sequences was accomplished with redox-active probes. As noted in Methods, the sensors can work without an added redox-active compound, making use of the fact that it is possible to electrochemically detect guanine, a constituent of nucleic acids. For the viral detection work reported below, the target nucleic acid was allowed to hybridize with one or more probes. The probe-target hybrid was captured on electrode sensor via a capture probe labeled with biotin or other capture reagent. Following this the electrode was brought in contact with a solution containing an electrochemical mediator and the enzyme substrate. The electrons generated were carried to the electrode by the mediator and a current was generated, which was measured with our monitor (see diagrams in Methods).

We have completed research and development of the sensors as proposed for Phase I. This includes study, testing and improvement of each of the sensor components (carbon inks, colloidal gold coating, capture probes, and overall configuration of the test electrodes and sample wells).

We have generated disposable and inexpensive electrochemical DNA-probe biosensors with colloidal gold electrodes that make use of the electroactive gene-probes. These were generated using procedures outlined in the Methods.

We now have data for over two months of self life of all proprietary test components and reagents. Suitably long shelf life of commercial items such as droppers, RNA release reagents is guaranteed by the manufacturers, but will be further tested in Phase II and during Phase III manufacturing scale-up. Our assay which contains the cAu/NA sensors, the lysis, probe hybridization and HRP label reagents, and the positive and negative controls, all stored in the refrigerator at ca. 4 C continue to deliver good, steady test results.

Viral Detection Results

The Phase I program of research was designed to demonstrate detection of both hepatitis A and polio virus. The purpose of our work was to develop an electrochemical detection system, reagents and procedures for direct detection of RNA viruses. This research was on 1) electrochemical sequence detection monitors 2) hybridization, 3) probe sensitivity, 4) specimen processing, and 5) signal amplification. Results of our Phase I efforts on viral detection are described below.

For hepatitis A, we first demonstrated detection of a synthetic DNA 62-mer having the target hepatitis A sequence (Figure 7).

For polio virus, we first demonstrated detection of virus-specific complementary DNA clone (cDNA). In these studies, as described below, a simple sandwich hybridization assay method was used, with unoptimized probes for the polio cDNA. Probes labeled with electroactive enzymes were used so that captured target-probe hybrids would generate a detectable electrochemical signal upon addition of the enzyme substrate. As shown in Figure 8 we detected the polio cDNA target. As shown, only small background currents were measured in the absence of the targets.

Noncomplementary viral nucleic acid sequences were used as additional controls in follow-on experiments. Cross-testing the signal response when polio virus and hepatitis A sequences were introduced in tests with probes for hepatitis A or polio viral sequences, respectively, allowed us to

demonstrate that our approach has the requisite specificity, i.e., only complementary target and probes produce the hybridization that leads to generation of an electrical current.

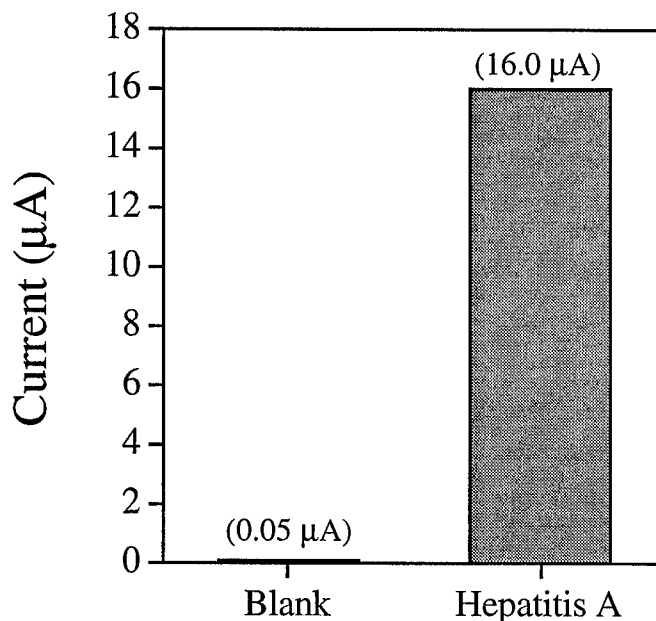


Figure 7. *Detection of Hepatitis A virus nucleic acid sequence with AndCare system. Synthesized target DNA for Hepatitis was assayed. In each case, a large current was measured when the target DNA hybridized to its complimentary probes (Test) and only small blank current was detected with non complementary probe (blank).*

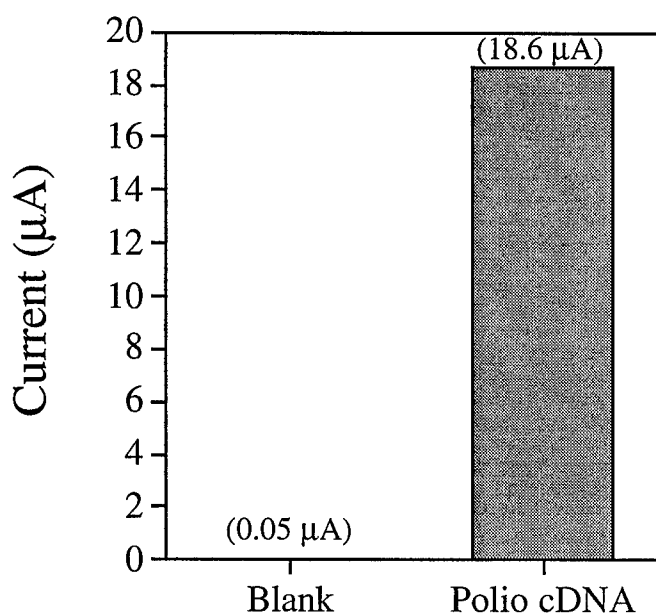


Figure 8. *Direct detection of polio virus cDNA. Polio virus cDNA was assayed with the AndCare system. A large current was measured when the target cDNA hybridized to its complimentary probes (Test) and only small blank current was detected with non-complementary probe (blank).*

Some preliminary experiments were done without probe or system optimization which showed the feasibility of detecting polio virus in crude samples, without target amplification. For this purpose we grew and harvested polio virus and used this material to demonstrate direct detection and quantification of polio virus in crude samples. In these studies, as described below, a simple sandwich hybridization assay method was used, with unoptimized probes labeled with electroactive enzymes. As in our other assays, only captured target-probe hybrids generate a detectable electrochemical signal upon addition of the enzyme substrate. For crude samples containing polio virus at 2.5×10^7 pfu/ml of polio virus was clearly detected. When experiments used 1 anti-DIG labeled detector probe and 1 capture probe, the average blank was $0.027 \mu\text{A}$ and the average signal was $0.205 \mu\text{A}$. Some experiments were run with multiple probes but these did not improve the signal to noise above that obtained with the single probe sets.

Related experiments were performed in which RNA was extracted from polio virus and then examined with our electrochemical detection assays, without amplification. Direct detection of the un-amplified RNA was shown. Typical results with RNA extracted from 5.7×10^7 pfu/ml of polio virus were $0.32 \mu\text{A}$ signal, $0.17 \mu\text{A}$ blank, with an average signal to blank ratio of 2.1. Direct RNA detection in these experiments has, surprisingly, a poorer signal to noise ratio than was obtained in the direct assays using crude samples reported above. Better results for direct detection without amplification are anticipated in further work, since this detection was obtained with an un-optimized system and single probe sets.

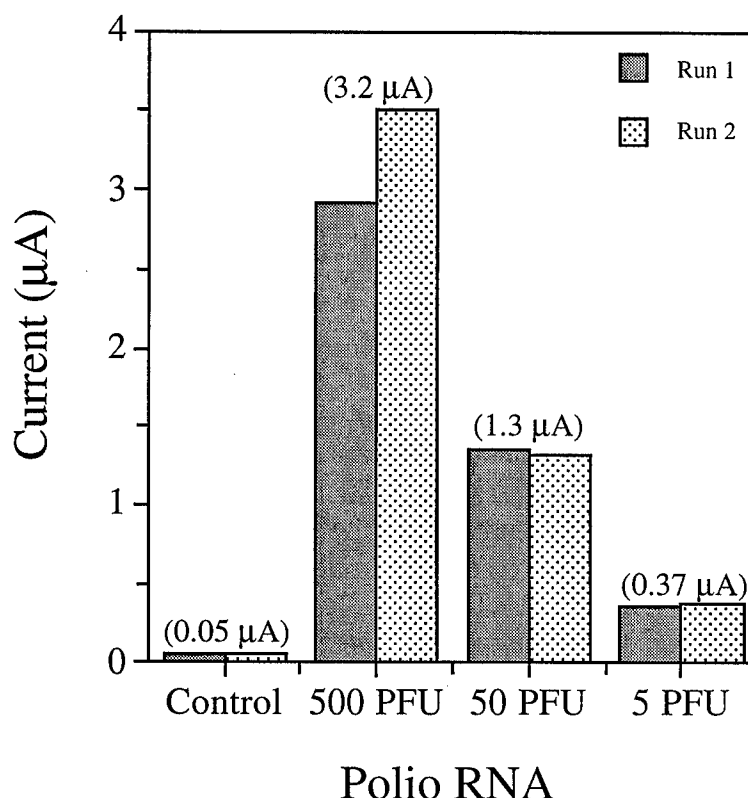


Figure 9. Detection of polio virus at low levels. Polio virus RNA was isolated from polio infected cells and 5, 50 and 500 pfu of viral RNA was amplified by RT-PCR. The PCR product was measured with the AndCare system. Large currents proportional to

viral RNA content were measured and only small background current was detected with RNA isolated from uninfected cells (control).

In order to demonstrate an extremely sensitive assay for polio virus using the AndCare system, we coupled our electrochemical detection system to a conventional PCR target amplification system. We grew and harvested polio virus, isolated RNA and used this material as the target. In these proof of principle experiments, polio virus at clinically relevant levels (5-500 pfu) was detected using our electrochemical detection system and reverse transcriptase polynuclease chain reaction (RT-PCR). The signals generated were proportional to the RNA levels (Figure 9), Electrophoresis verified that the PCR product was of the expected size.

As shown above, our electrochemical detection system can readily detect viral RT-PCR products at sensitivity levels that are clinically relevant. This is a dramatic advance, indicating that our system could nicely replace electrophoresis as a PCR detection method. Electrophoresis requires additional equipment and is relatively more time consuming and cumbersome. Since very simple labels are used (Biotin and Fluorescein) this approach could be applied to detect PCR amplification of any desired viral sequence. Concern that the RT-PCR product generated might not be the one desired led us to generate a two-step approach to verify specificity of product formation. In this two-step detection, a single sensor can be used to first monitor the PCR product formation, and second verify that it is the desired product by formation of a sandwich hybrid when a second probe is added. Briefly, the generalized RT-PCR process yields a product that is biotin labeled on one strand and fluorescein labeled on the other. This can be detected electrochemically. After detection, the fluorescein labeled strand can be washed off and the captured (biotin-labeled) strand can be verified to be the desired product by formation of a hybrid with a second, fluorescein-labeled probe. A current above background will be generated only if this second sandwich hybrid forms. Table I which follows presents conclusive evidence not only of RT-PCR product detection but also of the desired specificity of viral detection.

Some aspects of Table I merit further description. As noted above, RT-PCR reactions targeting viral RNA or polio virus plasmid and containing both a biotin and fluorescein-labeled primer generated a strong positive electrochemical signal. Reactions containing a biotin-labeled primer and an un-labeled second primer produced amplified products, as evidenced by the expected electrophoretic band, but gave no electrochemical signal (as expected). The absence of detectable electrophoretic bands in experiment 4 where formation of the second hybrid gave a significant electrochemical signal re-confirms that the sensor assay is much more sensitive than visual inspection of ethidium bromide fluorescence in an agarose electrophoretic gel. No signal was observed in the sample from which reverse transcriptase was omitted, confirming that the product of the RT-PCR reaction was in fact RNA and not a contaminating DNA intermediate. Experiments 1,2 and 6 dramatically illustrate the efficiency of the protocol used to remove the complementary (fluorescein-labeled) strand of the duplex prior to formation of the second hybrid with an added fluorescein-labeled probe. Assays of these samples without addition of the second probe gave signals approaching background levels.

Additional experiments were conducted in order to determine the specificity achievable with our electrochemical approach to detection of nucleic acid hybrids. For these experiments, a target DNA (sixty bases in length) was synthesized that was only partially complementary to the probe sequence (19 bases in length) and the generation of electrochemical signal was measured for this

Table I

Sample	Modified Primers in RT-PCR	input PFU	PFU/mL	mA One-step	mA (-) probe (- Fl strand)	mA (+) probe Hybridization Two-step	Gel Band
1. Viral RNA	Fl/Bio	500	5x10 ⁵	0.012	0.038	0.35	+
				2.23	0.038	0.402	
				2.471	0.038	0.386	
2. Viral RNA	Fl/Bio	50	5x10 ⁴	1.486	0.042	0.336	+
				1.349	0.048	0.208	
				1.344	0.095	0.258	
3. Viral RNA	Bio only	500	5x10 ⁵	0.033	0.028	0.277	+
				0.032	0.031	0.249	
				0.028	0.266	0.277	
4. Viral RNA	Bio only	50	5x10 ⁴	0.038	0.986	0.117	-
				0.033	0.068	0.135	
				0.034	0.141	0.121	
5. Viral RNA-RT	Fl/Bio	500	5x10 ⁵	0.035	0.021	0.037	-
				0.042	0.034	0.033	
				0.054	0.033	0.051	
6. Plasmid	Fl/Bio		~ 10 ⁷	2.339	0.034	0.543/0.616	+
				2.164	0.033	0.754	
				2.234	0.041	0.708	
7. No target	Fl/Bio			0.029	0.076	0.031	-
				0.036	0.105	0.027	
				0.029	0.039	0.098	
8. Uninfected RNA	Fl/Bio	"500"		0.036	0.049	0.043	-
				0.031	0.053	0.016	
				0.029	0.045	0.018	

--Reverse Transcriptase was omitted from RT-PCR reaction #5 in order to prove that signals are coming from amplified RNA and not a DNA intermediate of viral replication. As described in text, One-step Detection results from capture of Fluorescein-Biotin (Fl/Bio) hybrids at the gold electrode. Two-step Detection occurs after the Fl-labeled strand is washed away and a new Fl-labeled probe specific for the target is bound. RT-PCR Conditions are described in Methods.

test system. The synthetic target had only a single base mismatch (T(G). The result was compared to hybridization of the target with a probe that was fully complementary with the target. As shown in Figure 10, hybridization of complementary target was positive for hybridization and generation of an electrical current. The single base pair mismatch between target and probe resulted in a greatly reduced signal. Hybridization between the complementary probe and target generates a signal that increases linearly with the target concentration over the tested range of target from 0 to 10 nM; the small signal generated for the altered (one-base mismatched) target is unaffected by the target concentration over the same range (i.e. the small signal detected does not change with target concentration).

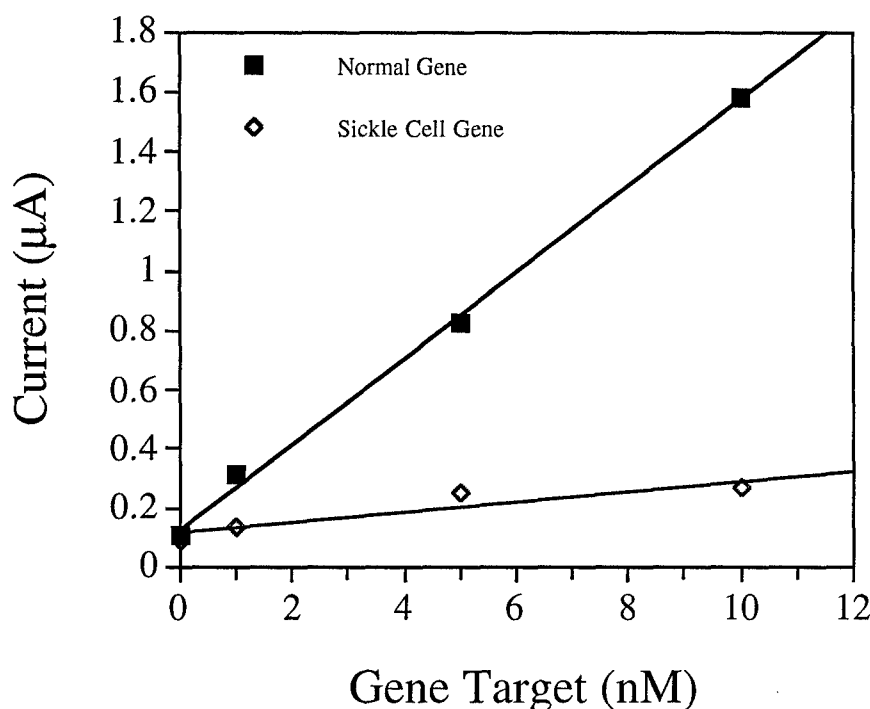


Figure 10. Demonstration of high specificity of the AndCare assay. The experiment contrasts the signals resulting from target hybridization with complementary probe sequences and non-complementary probe sequences where these differ by a single base pair mismatch. A large current was measured when the target hybridized to its complementary probe but little or no current was detected when probe had a single base mismatch.

5.0 CONCLUSIONS

During Phase I, the research team achieved six significant milestones:

1. Development of disposable and inexpensive electrochemical biosensors and monitors for detection of nucleic acid sequences specific to pathogenic viruses,
2. Good electrochemical detection of synthesized DNA complementary to hepatitis A virus,

3. Good electrochemical detection of polio virus complementary DNA (cDNA),
4. Demonstration of a sensitive assay for polio virus at clinically relevant levels using RT-PCR,
5. Culture of polio virus and rotavirus for further study, and
6. Demonstration of selectivity, so that point mutations in nucleic acid sequence are detectable.

6.0 REFERENCES

- Bosch, A., Gajardo, R., Diez, J.M., and Pinto, R.M. (1996) "Non isotopic automatable molecular procedures for the detection of enteroviruses", *Molecular and Cellular Probes*, **10**, 81-89.
- Crumbliss, A.L., Stonehuerner, J., Henkens, R.W., Zhao, J. and O'Daly, J.P. (1993) "A Carrageenan Hydrogel Stabilized Colloidal Gold Multi-Enzyme Biosensor Electrode Utilizing Immobilized Horseradish Peroxidase and Cholesterol Oxidase/Cholesterol Esterase to Detect Cholesterol in Serum and Whole Blood," *Biosensors & Bioelectronics*, **8**, 331-337.
- Henkens, R.W., Johnston, T.E., Nevoret, M.L., O'Daly, J.P., Stonehuerner, J.G. and Zhao, J. (1992a) Determination of lead by its inhibition of isocitrate dehydrogenase and diagnosis of lead poisoning, *Metal Ions in Biology and Medicine*, Eds. J. Anastassopoulou, Ph. Collery, J.C. Etienne, and Th. Theophanides, John Libbey Eurotext, Paris, **2**, 275-280.
- Henkens, R.W., Zhao J. and O'Daly, J.P. (1992b) Convenient enzymatic determination of trace mercury in water, *Metal Ions in Biology and Medicine*, Eds. J. Anastassopoulou, Ph. Collery, J.C. Etienne, and Th. Theophanides, John Libbey Eurotext, Paris, **2**, 317-318.
- Henkens, R.W. "Detection of Microbial Pathogens Using Gene-Probe Sensors," Scientific Conference on Chemical and Biological Defense Research, Edgewood Area Conference Center, Aberdeen Proving Ground, Maryland, 19-22 November 1996.
- Henkens, R.W., O'Daly, J.P., Wojciechowski, M., Zhang, Honghua., Naser N., Roe, R.M., Stewart, T.N., Wegner, S.W. (1997) "Electrochemical Detection of Nucleic Acid Sequences," patent pending.
- Ichimura, H., Tamura, I., Yamada, O., et al. (1992) "Hepatitis C virus RNA and hepatitis C virus antibody in the serum of patients with abnormal liver function," *J. Infect.*, **235**, 47-53.
- Jansen, R.W., Siegl, G., Lemon, S.M. (1990) "Molecular epidemiology of human hepatitis A virus defined by an antigen-capture polymerase chain reaction method," *Proc Nat'l Acad Sci USA*, **87**, 2867-71.

- Kaneko, S., Miller, R.H., Feinstone, S.M., et al. (1989) "Detection of serum hepatitis B virus DNA in patients with chronic hepatitis using the polymerase chain reaction assay," *Proc Nat'l Acad Sci USA*, **86**, 312-6.
- O'Daly, J.P., Zhao, J., Brown, P.A. and Henkens, R.W. (1992) "Electrochemical enzyme immunoassay for detection of toxic substances," *Enzyme Microb. Technol.*, **14**, 299-302.
- Ray, C.G. (1979) "An infectious disease viewpoint of diagnostic virology" in *Diagnosis of Viral Infections: The Role of the Clinical Laboratory* (Lenette, D., Specter, S., and Thomas K. eds.) University Press: Baltimore, 241-248.
- Robart, H.A. (1990) "Enzymatic RNA amplification of the enteroviruses," *J. Clin. Microbiol.*, **28**, 432-8.
- Zhao, J., Henkens, R.W., Stonehuerner, J.G., O'Daly, J.P., and Crumbliss, A.L. (1992) "Direct electron transfer at horseradish peroxidase/colloidal gold modified electrodes," *J. Electroanal. Chem.*, **327**, 109-19.
- Zhao, J., Henkens, R.W., Crumbliss, A.L. (1996) "Mediator-free Amperometric Determination of Toxic Substances Based on their Inhibition of Immobilized Horseradish Peroxidase," *Biotechnology Progress*, **12**:703-708.

7.0 BIBLIOGRAPHY

Publications

Henkens, R.W., O'Daly, J.P., Wojciechowski, M., Zhang, Honghua., Naser N., Roe, R.M., Stewart, T.N., Wegner, S.W. (1997) "Electrochemical Detection of Nucleic Acid Sequences," patent pending.

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